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Comparison of tumour morphology and structure from U87 and U118 glioma cells cultured on chicken embryo chorioallantoic membrane

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Abstract

The objective of this study was the morphological and structural characterisation of glioblastoma multiforme grown *in ovo*. Glioma cells U87 and U118 were implanted in the chorioallantoic membrane (CAM) of chicken egg. After 10 days of incubation, tumours were resected for further analyses. Culturing two types of glioblastoma tumours from U87 and U118 cell lines has shown a number of differences in their morphology, histology, and ultrastructure. CAM assay proved to be a useful tool for studying glioblastoma growth. The model provides an excellent alternative to current rodent models and could serve as a pre-clinical screening assay for anticancer molecules. It might increase the speed and efficacy of the development of new drugs for the treatment of glioblastoma.

Key words: extended glioblastoma multiforme, U87 and U118 cells, chorioallantoic membrane model.

Introduction

Glioblastoma multiforme (GBM) is the most common and most aggressive primary malignant brain tumour. It originates from glial cells and is characterised by infiltrative growth and intensive migration (5). GBM tends to spread quickly within the brain and nervous system but rarely spreads outside it. It usually penetrates deeply into the brain, making the tumour's entire removal very difficult. The main features of GBM are cell polymorphism and mitotic activity, vascular abnormalities, and remarkable necrotic foci (1).

The development of new anti-glioma strategies requires the use of appropriate models to evaluate the effect of tumours. *In vivo* glioblastoma models are based on the inoculation of glioma cells into the rodent brain or the use of transgenic mice causing spontaneous tumours. However, these models are weak because they

are characterised by variable growth rates and poor penetration, and it is also difficult to obtain morphological data (3, 4). The chicken embryo chorioallantoic membrane (CAM) model is a well-established method to keep explanted material alive and supply it with oxygen and nourishment (1). This model provides a useful tool to study the effects of molecules, which interfere with experimental tumours derived from cancer cell lines (2).

Glioblastomas are very heterogenic in their biological and morphological features. It was assumed that the tumours derived from two different glioma cell lines are characterised by different morphology, structures, and mitotic activity. The objective of this study was to evaluate the morphological and structural CAM differences between U87 and U118 cultured tumours.

Material and Methods

Glioma cells. Human glioblastoma U87 and U118 cell lines were obtained from the American Type Culture Collection (ATTC, Manassas, USA) and maintained in DMEM (Sigma-Aldrich Corporation, USA) supplemented with 10% foetal bovine serum (Sigma-Aldrich) and 1% penicillin and streptomycin (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂/95% air (NUAire DH Autoflow CO₂ Air-Jacked Incubator).

Chicken embryos. The fertilised eggs (*Gallus gallus*) of meat breed Ross 308 (n = 60 for one glioma cell line) were supplied from a commercial hatchery. The eggs were incubated in the incubator ALMD-1N3-7 with automatic egg rotation system (one full rotation per hour) at 37°C and 70% humidity.

Culture of GMB cells on a chorioallantoic membrane. Eggs were divided into two groups of 60: U87 group and U118 group. After seven days of egg incubation, the silicone ring with the deposited $3-4 \times 10^6$ U87 or U118 glioma cells suspended in 30 µL of culture medium was placed on the chorioallantoic membrane (CAM) in the area of formed blood vessels. The eggs were incubated for 10 days, and then the tumours were resected for further analysis. Forty-nine U87 and 24 U118 tumours were used.

Cell morphology. U87 and U118 cells were plated on 6-well plates (1×10^5 cells per well) and incubated for 24 h. The cells were stained with May-Grünwald Giemsa method (Sigma-Aldrich). The morphology was recorded under an optical microscope (DM750; Leica Microsystems GmbH, Wetzlar, Germany) using LAS EZ version 2.0 software.

Measurement and calculation of tumour volumes. The stereo microscope (SZX10, CellID software version 3.1; Olympus Corporation, Japan) was used to take digital photos of tumour. The measurements were taken with cellSens Dimension Desktop version 1.3 (Olympus). The tumour volumes were calculated with the following equation (9):

$$V = \frac{4}{3}\pi r^3 \text{ where } r = \frac{1}{2} \sqrt{\text{diameter 1} \times \text{diameter 2}}$$

Histological and immunochemistry image analysis. After resection, tumours were fixed in 10% buffered formalin (Sigma-Aldrich). Samples were dehydrated and embedded in paraffin (Sigma-Aldrich). Sections of 5 µm were mounted on poly-L-lysine-coated slides (Equimed, Poland) and stained with haematoxylin and eosin. For immunohistochemical analysis, proliferating cell nuclear antigen antibody (PCNA, DAKO M0879) in dilution 1:200 was used. PCNA-positive cells were visualized with Dako EnVision+System-HRP (Dako K 4010, Dako A/S, Denmark).

Cells and tissues were measured using a Nikon ECLIPSE 90i microscope coupled with a digital camera Nikon DS5-U1 and NIS-Elements AR microscope imaging software (Nikon Corporation, Japan). Morphometric evaluation and image analysis were performed using 20 measurements of each sample at 400 × magnification. The total number of cells and PCNA positive cells were counted in visual fields (40 µm²) of the tissue. Mitotic index was assessed as the number of mitotic figures in 10 visual fields.

TEM analysis. Tumour tissues were cut immediately after dissection into pieces of about 1 mm³ and fixed in a 2.5% glutaraldehyde solution (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.9). The samples were then rinsed in the same buffer, and transferred to a 1% osmium tetroxide solution (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.9) for 1 h. Subsequently, the samples were rinsed in distilled water, dehydrated in ethanol gradients, and impregnated with epoxy embedding resin (Fluka Epoxy Embedding Medium Kit; Sigma-Aldrich). On the next day, the samples were embedded in the same resin and baked for 24 h at 36°C. The blocks were then transferred to a 60°C incubator and baked for another 24 h. The blocks were cut into ultrathin sections (50–80 nm) using an ultramicrotome (Ultratome III; LKB Products, Sweden) and transferred onto copper grids, 200 mesh (Agar Scientific Ltd, United Kingdom). Subsequently, the sections were contrasted using uranyl acetate dihydrate (Sigma Aldrich) and lead citrate (lead (II) citrate tribasic trihydrate; Sigma-Aldrich). The sections were examined under a JEM-1220 transmission electron microscope (TEM) (JEOL, Japan).

Statistical analysis. The data was analysed statistically by monofactorial analysis of variance, and the differences between groups were tested by multiple range Duncan test using Statistica version 10.0 (StatSoft, USA). Differences with P < 0.05 were considered significant.

Results

Cell morphology. Both lines had different rates of growth and morphology (Fig. 1). U87 cells grew faster; they were bigger and had longer protrusions than U118 cells. They continued to grow and formed multilayer spheroids at a high density, while U118 formed monolayers without spheroids and peeled off the plate, and died at a high density.

Tumour morphology. The glioblastoma invaded CAM along its vessels (Fig. 2). In many cases tumours were observed outside the silicone ring. U87 and U118 tumours had oval shape and visible blood vessels on the surface (Fig. 3). U87 tumours were bigger and had more visible vessels than U118 tumours (Table 1).

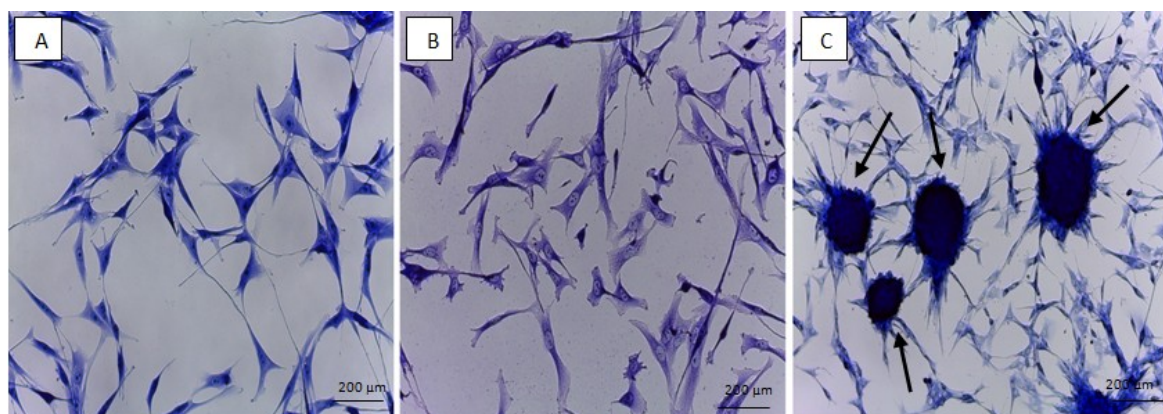


Fig. 1. Glioma cell lines: A, C - U87 glioma cell line; B - U118 glioma cell line. Arrows point to spheroids created by U87 cells

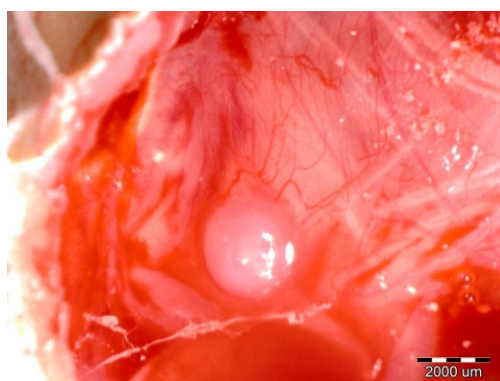


Fig. 2. Glioblastoma multiforme grown on the chorioallantoic membrane. Scale bar: 200 µm

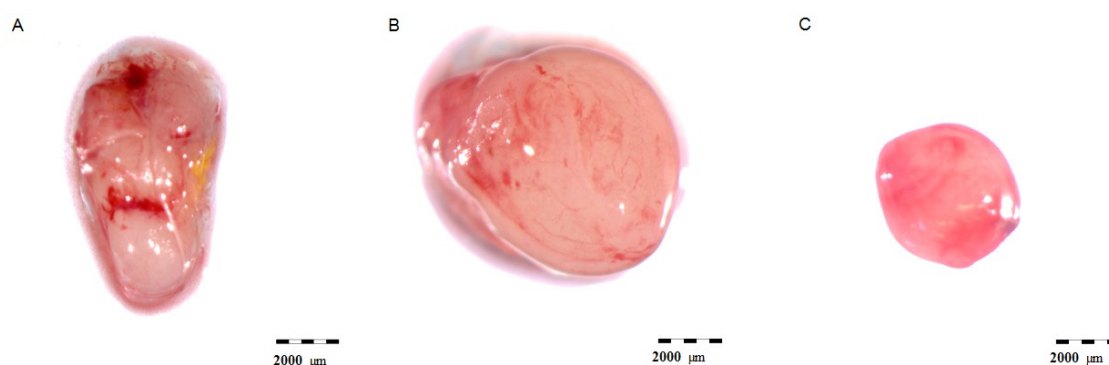


Fig. 3. Isolated tumours. A, B - 87 tumour, C - 118 tumour. Scale bar: 200 µm

Table 1. Characteristics of U87 and U118 glioma multiforme tumours

Parameter	Group		ANOVA	
	U87 tumour	U118 tumour	P-value	SE – pooled
Volume (mm ³)	47.4	15.1	0.0011	6.76
Average of number of glioma cells (on 40 µm ² area)	164	119	0.0000	5.6
Average of number of PCNA positive cells (on 40 µm ² area)	136	84	0.0025	6.1
Mitotic index	9.29	4.33		

ANOVA - analysis of variance; SE – pooled standard error

Histology and immunochemistry. In both tumours, the cell density was significantly higher than in the human brain nerve tissue (1). Size and shape of U87 and U118 cells revealed high polymorphisms. The picture of gliomas was characterised by the presence of different cells with small and larger atypical nucleus and high ratio of nucleus to cytoplasm (Fig. 4). The histological analysis revealed the presence of the multinucleated giant cells. In comparison to U87, the U118 tumour had a less compact structure and smaller amounts of cells in the tumour tissue (Table 1). Both tumours showed high mitotic activity; the mitotic index varied from 4.3 in U118 to 9.3 in U87 cells. Furthermore, PCNA staining showed a high proliferative activity of tumour: 74.6 % of U87 cells and 61.7% of U118 cells were PCNA positive (Fig. 5). Both cultured tumours showed a lot of features

characteristic for human glioblastoma: nuclear atypia, mitotic cells, and cellular variety.

TEM analysis of tumour. Fibroblast cells had elongated bodies, a rough endoplasmic reticulum (R.E.R), vacuoles, and groups of endocytotic vesicles. Most of the cells of both tumours had a high rate of protein synthesis what was confirmed by the highly developed R.E.R. The nuclei were elongated and had irregular shape, and unevenly distributed chromatin. A part of the nuclei contained spheroid bodies composed of granular material. Each cell line had mitochondria, which varied in size and shape, and were usually oval or elongated. U118 tumour cells were smaller in comparison to U87 cells, had a higher density of cytoplasm and a greater number of endocytotic vesicles (Fig. 6).

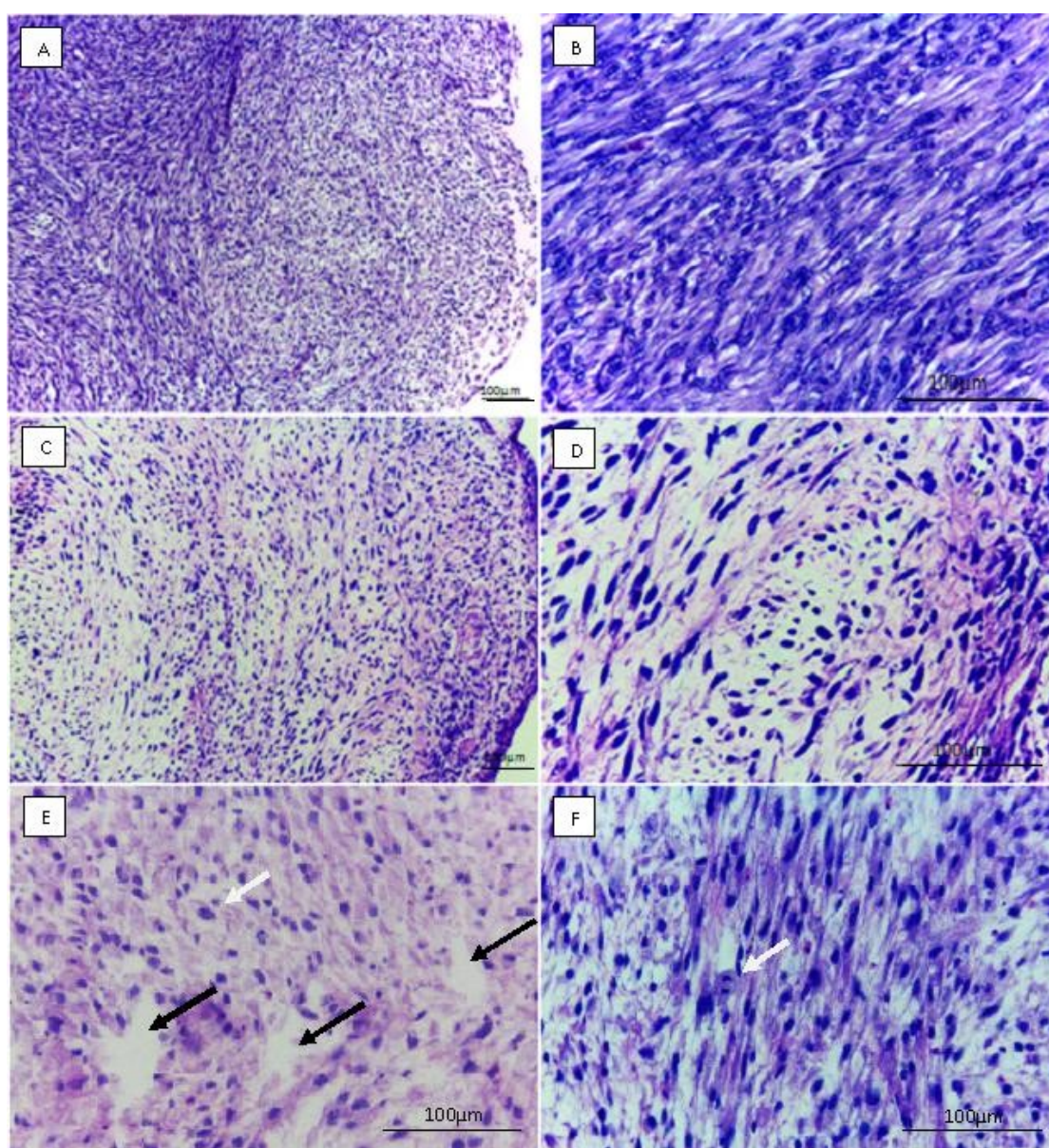


Fig. 4. Histology of glioblastoma multiforme tumour cultured on chorioallantoic membrane: A, B, E, F - U87 tumour, C, D - U118 tumour. Black arrows point to blood vessels; white arrows show mitotic figures. Scale bar: 100 µm

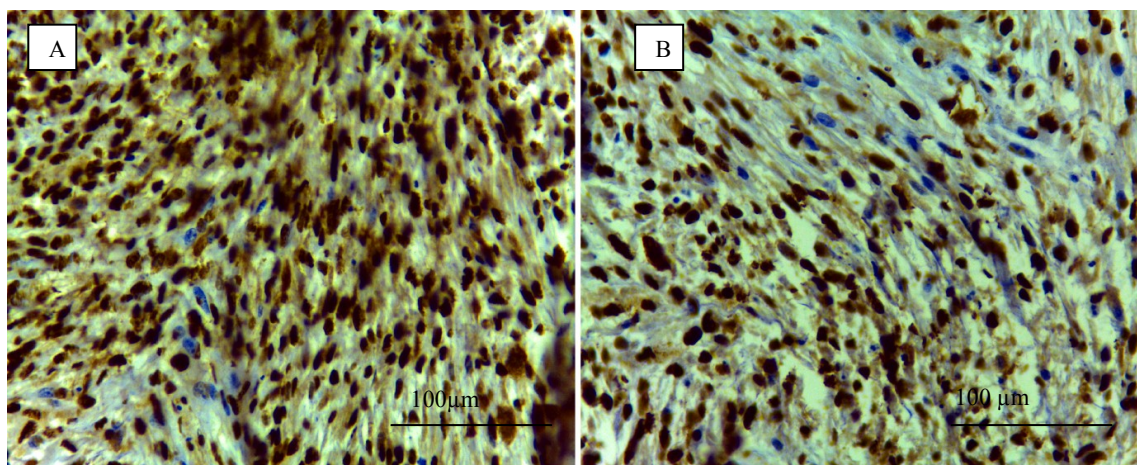


Fig. 5. Immunoperoxidase staining of PCNA: A - U87 tumour, B - U118 tumour. Brown nuclei represent PCNA positive cells, blue – PCNA negative cells. Scale bar: 100 µm

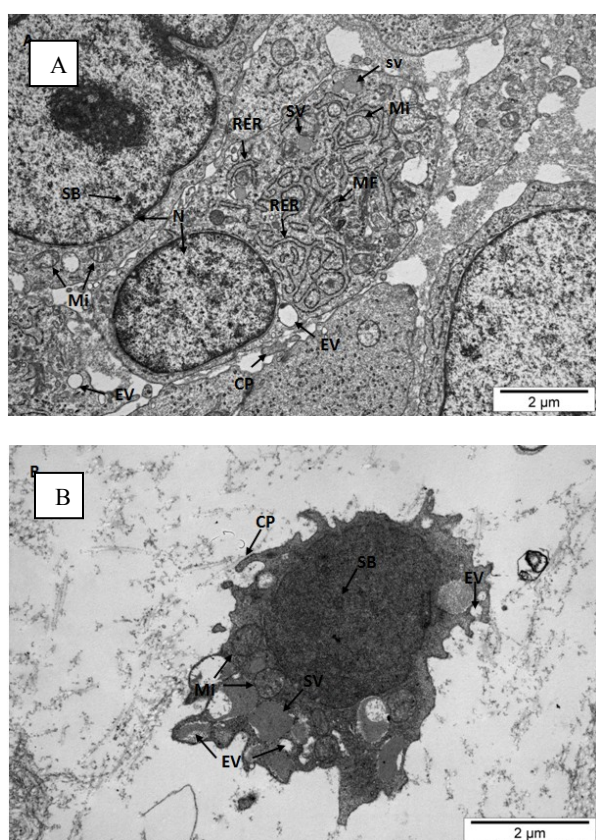


Fig. 6. Glioblastoma multiforme ultrastructure: A - U87, B - U118. CP – glioblastoma cell processes, EV – endocytotic vesicle, MF – myelin figures, Mi – mitochondrion, N – nucleus, RER – rough endoplasmic reticulum, SV – secretory vesicle, SB – spherical bodies. Scale bar: 2 µm

Discussion

The successful tumour therapy depends on a preclinical model, which can accurately reflect the growth and development of a tumour. Many studies are conducted with one-layer *in vitro* cultures, which have different morphology and physiology to a tumour. However, some cancer lines, *i.e.* glioma U87 cells, are able to create multi-layer spheroids (8).

The chick CAM assay is a commonly used method for *in vivo* evaluation of tumour growth (6). In comparison to other animal models, based on cell implementation in rat brains or use of transgenic mice, the CAM model allows more accurate observation of tumour growth, especially when treating it with tested substances. A resection of the entire tumour is also possible (11, 13). This is more effective in developing tumours than a rodent model; compared to the rodent model, the CAM assay is much faster, allowing the observation of a tumour after a few days, while the rodent model requires much longer time, from one to four months (12).

Two human glioblastoma cell lines: U87 and U118, using the CAM, were grown. In the case of tumour creation from U118, the results are restricted only to one study by Durupt *et al.* (7), where implementation of both cell lines was successful; however, the U87 cell line had a better ability to create noticeable tumours, as previously demonstrated by Balciūniene *et al.* (1). For the U118 line, the intensity of tumour creation was significantly lower; hence, only 40% of inoculated eggs developed a tumour. Although both cell lines were visible within 7 d of inoculation, the tumours were resected on the 10th d when they were larger. The similar observation was noted by Grodzik *et al.* (9). In many cases, tumours were observed outside the silicone ring, as previously demonstrated by Szmidt *et al.* (14). All of the formed tumours possessed the ability to penetrate the membrane and were characterised by an invasive growth and strong angiogenic response. Culturing two types of glioblastoma tumours from U87 and U118 cell lines revealed a lot of differences in their morphology, histology, and ultrastructure; U87 tumours were larger and had more vessels. In this study, they were almost three times larger than U118 line tumours; however, compared to the results by Grodzik *et al.* (9), they were half the size of U87 tumours. U87 line cells stucked closely to each other and filled free spaces in a tumour, while U118 cells were loosely arranged. However, the

cell density in both cases was much higher than in the normal human brain nerve tissue (1). Furthermore, the histological analysis of tumours revealed the presence of multinucleated giant cells. Their characteristic features are large sizes with nuclei of variable number, size and shape (15). In this study, histological characteristics of GBM grown *in ovo* demonstrated several typical human-like features: high proliferation, cellular variety, nuclear atypia, and even mitotic cells, similarly to the results obtained by Hagedorn *et al.* (10). The present CAM model allows the growth of a GBM with features similar to human tumours. The model is simple and fast, and can be useful in the research of GBM, as well as other tumours.

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